The impact of partial and complete loss of function mutations in endothelial lipase on hdl levels and functionality in humans

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Abstract: BACKGROUND: -Endothelial lipase is a phospholipase with activity against high density lipoprotein. Although a small number of mutations in LIPG have been described, the role of LIPG in protection against atherosclerosis is unclear. METHODS AND RESULTS: -We identified eight loss-of-function (LOF) mutations in LIPG in individuals with high HDL-C. Functional analysis confirmed that most rare mutations abolish lipase activity in vitro, indicating complete LOF (CLOF), while two more common mutations N396S and R476W reduce activity by 50%, indicating partial LOF (PLOF), and implying 50% and 75% remaining EL function in heterozygous CLOF and PLOF mutation carriers respectively. CLOF mutation carriers had significantly higher plasma HDL-C levels compared to PLOF mutation carriers. Apo-B depleted serum from CLOF carriers showed significantly enhanced cholesterol efflux acceptor capacity, whereas only trends were observed in PLOF carriers. Carriers of LIPG mutations exhibited trends toward reduced CAD in four independent cohorts (meta-analysis OR=0.7, p=0.04). CONCLUSIONS: -Our data suggest that the impact of LIPG mutations is directly related to their effect on EL function, and support that antagonism of EL function improves cardioprotection.

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The Impact of Partial and Complete Loss of Function Mutations in Endothelial Lipase on HDL Levels and Functionality in Humans

Running title: *Singaraja et al., The impact of LIPG on HDL levels and functionality*

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Abstract:

Background - Endothelial lipase is a phospholipase with activity against high density lipoprotein. Although a small number of mutations in *LIPG* have been described, the role of *LIPG* in protection against atherosclerosis is unclear.

Methods and Results - We identified eight loss-of-function (LOF) mutations in *LIPG* in individuals with high HDL-C. Functional analysis confirmed that most rare mutations abolish lipase activity *in vitro,* indicating complete LOF (CLOF), while two more common mutations N396S and R476W reduce activity by ~50%, indicating partial LOF (PLOF), and implying ~50% and ~75% remaining EL function in heterozygous CLOF and PLOF mutation carriers respectively. CLOF mutation carriers had significantly higher plasma HDL-C levels compared to PLOF mutation carriers. Apo-B depleted serum from CLOF carriers showed significantly enhanced cholesterol efflux acceptor capacity, whereas only trends were observed in PLOF carriers. Carriers of *LIPG* mutations exhibited trends toward reduced CAD in four independent cohorts (meta-analysis OR=0.7, p=0.04).

Conclusions - Our data suggest that the impact of *LIPG* mutations is directly related to their effect on EL function, and support that antagonism of EL function improves cardioprotection.

Key words: genetics; high-density lipoprotein cholesterol; lipids; cardiovascular disease

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Introduction

Reduction of low density lipoprotein cholesterol (LDL-C) has long been a primary target of coronary artery disease (CAD) prevention. However, despite lowering LDL-C with statins, a significant residual risk of CAD still remains, emphasizing the need for novel therapies (1). Plasma high-density lipoprotein cholesterol (HDL-C) is an independent risk factor and is inversely associated with CAD (2). The protective function of HDL-C in CAD is postulated at least in part to arise from its role in reverse cholesterol transport, the process whereby excess cellular cholesterol is transported to the liver for fecal excretion, and through its anti-inflammatory, anti-oxidative and anti-apoptotic functions (3).

Endothelial lipase (EL) is a member of the triglyceride lipase family that includes lipoprotein lipase (LPL) and hepatic lipase (HL). LPL primarily acts on TG-rich ApoB containing lipoproteins, HL acts on all classes of lipoproteins and EL acts preferentially on HDL lipids (4).

Studies of EL functionality in mice by overexpression and gene targeting show altered plasma HDL-C levels by affecting the fractional catabolic rate of HDL (5). One model suggests that EL hydrolyzes HDL phospholipids, generating smaller PL-depleted HDL particles that are more rapidly catabolized (6). Studies in humans also suggest that mutations that reduce EL activity correlate with increased plasma HDL-C levels (7, 8).

The role of EL in modulating risk for CAD is unclear. Male *Lipg*$^{-/-}$ mice show increased plasma HDL-C, phospholipid and LDL-C (9, 10) as well as increased HDL particle size (9). Associated with this, aortic lesions are reduced in *Lipg*$^{-/-}$ x *ApoE*$^{-/-}$ mice (9). However, aortic lesions are not changed in another model of *Lipg*$^{-/-}$ x *ApoE*$^{-/-}$ or in *Lipg*$^{-/-}$ x *Ldlr*$^{-/-}$ (10), and the reasons for this discrepancy are not readily apparent.

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It is unclear in humans whether loss of function of *LIPG* leading to raised HDL-C levels confers a decreased risk for atherosclerosis.

Here we have assessed the effects of CLOF and PLOF *LIPG* mutations on HDL-C levels, HDL functionality and risk for CAD. Individuals with heterozygous mutations in *LIPG* show significantly increased plasma HDL-C and improved HDL functionality. In addition, mutations in *LIPG* are associated with reduced atherosclerosis.

Results

Mutations in *LIPG* have discrete and diverse effects on EL function

We sequenced a cohort of 177 unrelated individuals with extreme high HDL-C (HDL-C≥90th percentile), in whom mutations in CETP, GALNT2, LCAT, LPL, APOAI and ABCA1 had been excluded, and identified 23 individuals with 8 different *LIPG* mutations, six of which are novel (Table 1) (Fig 1A). To determine if these mutations impact EL function, we utilized a fluorescent phospholipid substrate to quantify EL activity (11). All mutants showed significantly lower phospholipase activity compared with wt EL (Fig 1B). The *LIPG* mutations E391K, N396S and R476W showed partial loss of function (~50%). All the other mutations showed EL activity that was significantly different from wild-type and not different from mock transfected supernatants (CLOF) (Fig 1B). The 5′ UTR mutation M1-18T>C was not generated *in vitro* because a cDNA construct with a CMV promoter was used. Thus, *in vitro* data suggests that the catalytic domain of EL (amino acids 169-274) does not tolerate missense sequence changes. However, missense mutations in the c-terminal lipase domain of EL (amino acids 347-482) still retain partial activity *in vitro*. Thus, mutations in *LIPG* have variable impact on EL function.

Plasma HDL-C is highest in CLOF mutation carriers

Extrapolation of the observed *in vitro* effects of *LIPG* mutations to EL activity *in vivo* suggests

that heterozygous PLOF carriers will retain at least 75% of EL activity, whereas heterozygous CLOF carriers will retain at least 50% of EL activity. We extended the pedigrees of the probands to identify additional carriers of mutations in *LIPG* (Table 2), and assessed plasma HDL-C levels in mutation carriers (n=131) compared to unrelated population controls (n=756).

Carriers of *LIPG* mutations showed a significant 15.5mg/dL (28.7%) increase in HDL-C compared to unrelated controls (p=0.005; Table 3). Carriers of CLOF mutations showed a 23.2 mg/dL (42.9%) increase in plasma HDL-C, whereas carriers of PLOF mutations only showed a 13mg/dL (24.5%) increase (Table 4). No significant changes in plasma LDL-C, triglycerides or BMI were observed in carriers compared to controls (Table 3). No differences were observed in alcohol consumption, smoking, and history of diabetes or hypertension (data not shown).

Thus, the *in vitro* effects of various *LIPG* mutations correlated with the *in vivo* effects as measured by HDL-C levels. In our cohort, one individual was a compound heterozygote, carrying H220Q, a CLOF mutation, and N396S, a PLOF mutation. This individual would be expected to have only 25% of remaining EL activity. Consistent with this high level of EL inhibition, this individual showed a 54.6mg/dL (100.9%) increase in plasma HDL-C compared to controls (Table 4). In addition, 2 individuals were homozygotes for the PLOF mutation N396S (Table 4, Fig 1C).

Carriers of PLOF mutations show marked variability in HDL-C levels

We next assessed the variability of HDL-C levels in partial and complete LOF carriers. We defined high expressivity as those with HDL-C≥80th percentile after adjusting for age and sex, by comparing the HDL-C level of each carrier to those of age and sex matched individuals in the Lipid Research Clinic (LRC) database. A total of 83.4% of individuals with CLOF mutations had HDL-C levels greater than the 80th percentile, compared to only 69.6% of persons with the PLOF

mutation R476W and 62% of persons with the PLOF mutation N396S (N396S vs CLOF: p=0.03; R476W vs CLOF: p=ns; Fisher's exact test) (Fig 1D). These data indicate that N396S and R476W represent relatively mild mutations that show variable effects on HDL-C levels in humans.

Increased cholesterol efflux acceptor capacity in *LIPG* mutation carriers

HDL-C levels are correlated with susceptibility to atherosclerosis (2), but measures of HDL functionality show similar or even stronger correlation with CAD (12, 13). In addition, HDL functionality has effects on CAD independent of HDL-C levels (12). One aspect of HDL functionality, lipid efflux, may be a stronger predictor of CAD than HDL-C levels, and it was recently noted that every 1 standard deviation increase in lipid efflux was correlated with a significant ~25% decrease in CAD in humans (13).

Efflux of cholesterol from macrophages to ApoB-depleted serum from CLOF mutation carriers was significantly increased (% efflux: controls: 16.3±1.5 (n=4); CLOF: 19.6±2.4 (n=5), p=0.04) (Fig2A). This was less apparent in PLOF mutation carriers where efflux acceptor capacity was elevated but not significantly different from controls (PLOF: 17.7±2.5 (n=9), p=0.3) (Fig 2A).

The impact of anti-inflammatory and anti-oxidative effects of HDL on CAD has also recently been described (14). We determined if HDL from *LIPG* mutation carriers displayed these properties, by quantifying VCAM-1 expression and superoxide production. We found no significant differences in either of these parameters. However, a trend toward decreased (by 53%) superoxide production was observed in mutation carriers (Supplementary figure 1).

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LIPG mutation carriers have larger HDL particles and increased large HDL particle concentration

We next performed NMR analyses on HDL from *LIPG* mutation carriers. HDL from CLOF carriers was larger in size (CLOF: 10.2±0.6 nm, n=4; controls: 9.2±0.6 nm, n=6, p=0.04) (Fig 2B). No changes in LDL or VLDL particle sizes were observed (data not shown). The increase in HDL size was less apparent in PLOF carriers, and was not significantly different from controls (PLOF: 9.6±0.6, n=9, p=0.3). The concentration of large HDL particles was also significantly increased in CLOF carriers compared to both PLOF carriers and controls (CLOF: 12.6±3.2, n=6; controls: 5.1±2.9 μmol/L, n=4, p=0.006; PLOF: 8.6±3.5, n=9, p=0.04) (Fig 2C). No changes in concentration of VLDL and LDL particles were observed. The concentration of large particles in PLOF carriers was not significantly different from controls (p=0.1). Increased HDL particle size and increased large HDL particle concentration have been associated with reduced CAD (15, 16). HDL particles were also assayed using 2-D gel electrophoresis. A specific ~50% increase in α-1 HDL was observed in the CLOF carriers (CLOF: 21.7±4.8, n=4; controls: 14.5±3.8, n=5, p=0.047, Fig 2D), whereas no change was observed between PLOF carriers and controls (PLOF= 20.3±7.6, n=9, p=0.2).

HDL from _LIPG_ mutation carriers is enriched in phospholipids

The efficiency of lipid efflux acceptors depends on HDL phospholipid content (17, 18). We assessed the lipid composition of HDL isolated from *LIPG* mutation carriers. EL has high phospholipase activity toward HDL (4, 6). In agreement with this, HDL from carriers was enriched in phosphatidylcholine (PC) (controls: 541.2±187.7, n=4; CLOF: 695.4±132.2, n=6, mg/mL, p=0.16, Fig 2E). This data suggests that increased HDL phospholipid content in carriers result in improved efflux capacity. When PC subspecies were quantified, longer chain length

PCs (38-carbon) were significantly increased in HDL from CLOF compared to controls (CLOF= 156.6±37.8 mg/ml, n=6; controls= 96.1±39.2, n=4, p=0.04, Fig 2F). No significant difference was observed between PLOF carriers and controls. A trend to increased 36-carbon PCs was also observed in CLOF compared to PLOF carriers and controls. No differences in 34-carbon length PCs were observed irrespective of mutation strength (Fig 2F). These data suggest that EL acts preferentially on long chain PCs.

Assessment of atherosclerosis in *LIPG* mutation carriers

We assessed the impact of *LIPG* mutations on risk for CAD in the 131 family based mutation carriers compared to unrelated controls. A non-significant trend toward decreased CAD in mutation carriers was seen (carriers: 14/131 (10.7%) with CAD; unrelated controls: 111/756 (14.7%) with CAD, OR=0.69; 95%CI=0.39:1.25; p=0.28) (Table 5). Power calculations show we had 80% power to observe a significant association with CAD for an effect size of OR=0.37 or less, due to the low number of mutation carriers in this cohort. CLOF mutation carriers had lower CAD than either PLOF carriers or controls (CLOF: 2/22 (9.5%) with CAD; PLOF: 11/107 (13.2%) with CAD; controls: 111/756 (14.7%) with CAD, p=ns for all comparisons). In addition, the age at onset of CAD was significantly delayed in carriers of CLOF mutations compared to controls (CLOF: 72.0±4.2 yrs; PLOF: 64.6±12.7; controls: 51.1±11.7; CLOF vs controls: p=0.01; CLOF vs PLOF: p=ns).

To further extend these findings, we assessed CAD rates in mutation carriers in the Rotterdam cohort, a prospective, population based cohort (19). Although N396S and R476W are PLOF mutations, they are the most frequent mutations in Caucasians (Population frequency: N396S ~2.2%, R476W ~1%). Thus, these two mutations were genotyped in the Rotterdam cohort. As in the family based cohort, a non-significant decrease in CAD was observed in

mutation carriers (carriers: 17/156 (10.9%) with CAD; controls: 752/5658 (13.3%) with CAD, p=0.37, OR=0.79, 95%CI=0.48-1.32) (Table 5). CAD was defined as fatal or non-fatal myocardial infarct. The missing data rate was ~4% for this cohort.

We next selected two cohorts with high risk for CAD and therefore with greater power to assess the impact of *LIPG* mutations on CAD in populations of small size. Again, N396S and R476W, the two most frequent mutations were assessed, even though they showed only small effects on EL function. We first utilized the GiraFH cohort, a retrospective, multi-center cohort of 2068 individuals with familial hypercholesterolemia (FH) (20). In contrast to the family and Rotterdam cohorts in which the CAD rate was ~14%, the CAD rate in the GiraFH cohort was ~30% with an average age of onset of 48.8±10.9yrs. A trend towards reduced CAD that did not reach statistical significance was observed in mutation carriers (carriers: 11/57 (19.3%) with CAD; controls: 579/2011 (28.8%) with CAD, p=0.12, OR=0.59, 95%CI=0.31-1.15) (Table 5).

We next assessed the frequency of *LIPG* mutations in the PAS cohort, a cohort with premature atherosclerosis where cases were recruited as part of a prospective cohort with symptomatic CAD before the age of 51 years, defined as MI, coronary revascularization, or evidence of at least 70% stenosis in a major epicardial artery (21). DNA samples from blood donors at routine Sanquin Blood Bank donations were controls. If mutations in *LIPG* are associated with reduced CAD, then we expect lower mutation frequency in those with premature CAD. Indeed, the frequency of *LIPG* mutations in the PAS cohort was 2.1%, whereas the frequency of mutations in controls was 3.2% (p=0.2, OR=0.66, 95%CI=0.36-1.28) (Table 5).

Combined meta-analysis of the Rotterdam, GiraFH and PAS cohorts showed an OR of 0.7 with p=0.037 (95%CI=0.50-0.98) (Table 6). Thus, when cohorts were pooled, a reduction in CAD in *LIPG* mutation carriers is observed. However, underestimation of the impact of *LIPG*

mutations on atherosclerosis may have occurred since only the two PLOF *LIPG* mutations with small effects on HDL were assessed in three of the four cohorts.

Discussion

In this study, we have identified six novel and two previously described mutations in *LIPG* through sequencing of a cohort of high HDL-C individuals. We show that mutations in *LIPG* result in significantly increased plasma HDL-C and changes in HDL function compatible with cardioprotection. The effect of these mutations on HDL-C levels and HDL functionality is directly correlated with the impact of these mutations on EL activity. In addition, our data indicate that mutations in *LIPG* may confer protection against atherosclerosis.

Measures of HDL functionality show similar or even stronger correlation with CAD compared to that between HDL-C and CAD (12, 13). ApoB-depleted serum from individuals with CAD displays significantly reduced efflux acceptor capacity, confirming in humans that efflux capacity is robustly and inversely correlated with atherosclerosis (13). Using identical methodology, we found that ApoB-depleted serum from CLOF mutation carriers displayed significantly improved macrophage cholesterol efflux capacity. The phosphotidylcholine content of HDL particles is critical for efficiency of cholesterol efflux acceptors (17, 18). HDL isolated from CLOF mutation carriers showed increased phosphotidylcholine content, agreeing with the fact that EL is a phospholipase, and providing the mechanism underlying the improved efflux capacity. PLOF carriers showed trends towards improved efflux capacity. We assessed the impact of HDL on anti-oxidative and anti-inflammatory endothelial function (14), and found no significant effect of *LIPG* on these parameters. A non-significant 53% reduction in superoxide production was observed in mutation carriers, suggesting a beneficial effect of this HDL on anti-

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oxidative capacity. Overall, no significant adverse effects of *LIPG* reduction on HDL functionality were observed.

We next assessed the impact of *LIPG* mutations on CAD in humans. An important caveat is that many carriers of the two PLOF *LIPG* mutations, N396S and R476W, do not display elevated plasma HDL-C levels. Thus, if EL has an impact on atherosclerosis, and this is mediated through raised HDL-C, then utilizing only these mutations to assess the impact of EL on atherosclerosis will significantly undermine the effect. Despite this caveat, since these two mutations are by far the most frequent, we used them to assess the impact of *LIPG* on atherosclerosis.

Studies assessing the impact of genes modulating plasma HDL-C levels on atherosclerosis have been equivocal. One major factor contributing to this is the study of mild mutations with small effects on HDL-C levels as a surrogate for CLOF mutations. Selection of a few mutations with modest effects on protein function as a proxy for the inhibition of the majority of protein function often results in variable findings. However, in general, the more frequent mutations in populations tend to be mild LOF mutations, requiring the study of these in order to achieve adequate power.

Another factor significantly confounding such studies is the low risk for CAD in the general population. In addition, the baseline HDL-C levels from which elevation occurs significantly influence the impact of HDL-C on atherosclerosis. Increasing HDL-C from a baseline of 45mg/dL or greater does not provide much additional benefit against CAD (22, 23). Other lipoproteins such as LDL are also important, with greater impact on CAD being observed when HDL-C is increased in the presence of high LDL-C (22). These factors led us to select for further assessment, two cohorts with high atherosclerosis incidence, one with individuals with

familial hypercholesterolemia (GiraFH), and the other with individuals with premature atherosclerosis (PAS). An alternate strategy was to perform association studies in a very large unselected population cohort. However, because of the mild dysfunction of the two most common *LIPG* mutations, and the low rate of CAD in unselected populations, we chose to study selected cohorts.

We found that mutations in *LIPG* result in a trend toward protection against CAD in four different cohorts. When a combined meta analysis was performed, we observed a significant ~30% reduction in CAD in *LIPG* mutation carriers

The role of *LIPG* activity in atherosclerosis is unclear. Plasma EL concentrations are significantly increased in a cohort with metabolic syndrome and subclinical CHD (24), as well as in cultured endothelial cells in response to pro-inflammatory cytokines, and in humans during acute inflammation (25), suggesting a correlation between EL levels and the development of atherosclerosis. However, EL enhances the selective uptake of HDL-C though a SR-B1 mediated pathway (26), suggesting an atheroprotective role. In contrast, overexpression of EL resulted in a significant decrease in macrophage to feces reverse cholesterol transport (RCT) (27), although RCT was unchanged in *Lipg*$^{-/-}$ mice (28). Several human studies have assessed the impact of variants in *LIPG* on atherosclerosis. However, the majority of these studies assessed the variant 584C/T (T111I), which has no functional effect on LIPG (8) and thus is unlikely to modulate CAD risk. Two studies showed significantly reduced atherosclerosis in carriers of the T111I variant (29, 30), both independent of plasma HDL-C. However, since this variant does not influence EL activity directly (8), it is possible that the association with CAD resulted from this variant being in linkage disequilibrium with other loss of function *LIPG* mutations in these populations. A third study showed no impact of this variant on risk of CHD or on plasma HDL-C

levels (31) and a fourth showed no association between this variant and CAD, although carriers of the variant showed increased plasma HDL-C (32). The intronic variants C+42T/In5 and T+2864C/In8 were associated with fewer myocardial infarctions, although significance was lost after multiple testing (33). Two other SNPs in the 3'UTR of *LIPG* also showed no altered risk for CAD (34, 35). However, if these SNPs affect EL function is unknown.

Two studies thus far have assessed the association between a *LIPG* mutation known to compromise LIPG function and atherosclerosis. The first study assessed the association between the partial LOF N396S mutation and a surrogate marker for CAD, intima media thickness (IMT), and found no association, although plasma HDL-C levels were mildly increased (8). However, this study was performed in only four N396S mutation carriers and no conclusions can be reached from such a small study. The next study also assessed the impact of the mild N396S mutation on MI (36). Although >100,000 individuals from 20 different cohorts were assessed, overall, no impact of N396S on MI risk was observed (36). Numerous factors may have confounded this analysis. One crucial issue is HDL-C levels in the controls, and not just the magnitude of increase in carriers. The impact of HDL-C on cardiovascular risk is not linear and is curtailed in people with higher HDL-C levels (22, 23). Smaller increases from lower HDL levels confer larger impact on CAD risk compared to similar increases from higher baseline HDL (>45mg/dL) (22, 23). Another factor may be that the HDL-C increase was too small to detect an impact on MI. The authors used an HDL-C increase of 0.14mmol/L to predict the impact on MI, and for power calculations. However, of the 6 prospective cohorts, 3 had HDL-C increases of only 0.03, 0.04 and 0.08 mmol/L (the other 3 cohorts each had an increase of 0.14mmol/L), thereby perhaps resulting in an underpowered study. Another factor is that HDL-C levels are only reported for the 6 prospective cohorts. Thus, if HDL-C was increased in the 14

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case-control cohorts is unknown. Yet another factor may be the variety of ethnicities and environments represented by the 20 cohorts, and may explain the vastly varying impacts on MI for each cohort.

Recently, it was demonstrated that the mass and activity of EL isolated from individuals with cardiovascular disease was significantly higher than those without CAD (37), suggesting a direct correlation between EL activity and atherosclerosis. In addition, plasma EL activity was significantly correlated with coronary risk scores in the Framingham Study (37).

Our current study using human mutations does not model the almost complete inhibition of EL achievable with drugs. However, the major impact of homozygous and compound heterozygous mutations on plasma HDL-C, along with the increasing efflux functionality observed with decreasing EL activity suggests that inhibition of EL could be cardioprotective.

Materials and Methods:

Patients

Subjects were selected from the Lipid Clinic Network and Vascular Research Network (Amsterdam, Netherlands) and through the Centre for Molecular Medicine and Therapeutics (Vancouver, Canada). The main criterion was HDL-C$\geq$90th percentile, adjusted for age and gender, in unrelated probands. Families of unrelated probands were expanded, and detailed pedigrees generated. All individuals of families in which the high HDL proband had a mutation in *LIPG* were genotyped to assess segregation of the high HDL-C trait with *LIPG* mutations. All carriers of *LIPG* mutations were pooled, and compared to pooled non-carrier controls consisting of first-degree relatives of each mutation carrier. The Ethics Committees of the Academic Medical Center, Amsterdam and the University of British Columbia, Vancouver accepted the protocol for genetic analysis. All subjects signed informed consent forms.

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Fasting blood was drawn into EDTA-containing tubes and plasma and stored at -80°C. Leukocytes were isolated for DNA extraction. Lipoprotein measurements were performed as described (38).

DNA sequencing

DNA primers were designed to flank *LIPG* exons and adjacent intron boundaries as defined in the UCSC (hg18) release of the human genome (http://genome.ucsc.edu/). Reference DNA/Protein sequences used for sequencing and data analysis include NM_006033.2 and NP_006024. Primer sequences were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR products were amplified, purified using the AMPure kit (Agencourt, Beverly, MA), and sequenced by fluorescent dye-terminator chemistry (Seqwright, Houston TX). Known and novel SNPs in each gene were identified from sequence analysis using Sequencher v 4.9 (Ann Arbor, MI) and confirmed in dbSNP build 130 (NCBI, http://www.ncbi.nlm.nih.gov).

***In vitro* functional assessment of *LIPG* mutations**

All identified mutations were designated novel based on published literature and if absent from dbSNP (Build 130). Functional impacts were predicted using Polyphen 2.0 (http://genetics.bwh.harvard.edu/pph2/). All mutations except the promoter splice site Met1-18T>C mutation were generated in a *LIPG* cDNA clone (Origene) by site directed mutagenesis (Stratagene). Sense primers: Leu130Phe, cccaccagtttacacggatgcggtcaataatacc; Gly196Arg, ggtttggatcctgccaggcccatgtttgaagg; His220Gln, gcagattttgtggatgtcctccagacctacacg, Glu388Stop, ggaaatagtgtagcggatcgagcagaatgc; Glu391Lys, ggaaatagtggagcggatcaagcagaatgc; Asn396Ser, gcagaatgccaccagcaccttcctggtctacacc; Arg476Trp, gcatatccccaggctgggagctctggtttcg. Anti-sense primers were complementary to the sense primers. Positive clones were sequence confirmed. EL

protein was generated by Lipofectamine (Invitrogen) mediated transient transfection into HEK293T cells. 72 hours post-transfection, cells were incubated with 750U of heparin for 30 minutes at 37°C and media was concentrated, followed by centrifugation at 1500g for 30 minutes (4°C). Cleared supernatant was stored at -80°C. Cells in PBS were centrifuged at 800g for 5 minutes (4°C) and lysed in 100ul lysis buffer (0.025M ammonium chloride pH, 5mM EDTA, 0.4mg/mL SDS, 8.0mg/mL Triton-X 100 with proteinase inhibitor and heparin). Following sonication and centrifugation, supernatants were stored at -80°C. Proteins resolved on NuPage 12% BisTris gels (Invitrogen) were transferred to PVDF membranes and probed with antibodies to the N-terminus of EL (Abcam: ab14797). Bands were visualized with Supersignal Chemiluminescent Substrate (Thermo) on X-Ray Film (Thermo). Analyses of the lipase activity were performed using a fluorescent phospholipid substrate as described (11). A kinetic read was carried out for each sample, and a time-point was chosen in the linear range of the assay to ensure that the reduced activity of *LIPG* mutants did not arise from insufficient substrate.

HDL functionality assessments

Fasting plasma and serum was collected from *LIPG* mutation carriers and snap frozen. For lipid efflux studies, frozen sera were analyzed by Vascular Strategies LLC, using established protocols (13, 39). This assay quantifies total cholesterol efflux mediated by pathways of known relevance in macrophages (ABCA1 and G1, SR-BI, and aqueous diffusion). Briefly, J774 murine macrophages were labeled with 2μCi/mL ^{3}H cholesterol for 24 hours in the presence of ACAT inhibitor (Sandoz 58-035) and equilibrated overnight with 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP in the presence of ACAT inhibitor. ApoB-depleted serum was obtained by PEG precipitation. 2.8% v/v ApoB-depleted serum was used as efflux acceptor for 4 hours. 20 μg/ml apoA-I, 20 μg/ml HDL3, 2% v/v human serum and medium alone were used as controls. Efflux

was quantified by liquid scintillatio . For lipoprotein particle sizes and concentrations, proton NMR spectra of each plasma specimen (0.2 mL) were acquired in replicates using an automated 400-MHz lipoprotein analyzer at LipoScience (Raleigh, NC) following described protocols (40). For HDL subfraction analysis, 2-D gel electrophoretic separation was performed by Dr. Bela Asztalos using established protocols (41). HDL composition analysis was carried out by isolating the HDL particles using gradient gel electrophoresis (GGE) (Lipoprint ^{TM}, Quantimetrix, CA). In brief, 25 µl of plasma was loaded onto the gel following manufacturer's instructions. The gels were stained with Sudan Black to locate lipoprotein bands for excision. Excised lipoproteins were utilizing the Bligh and Dyer method (42) and analyzed by high-resolution liquid chromatography/mass spectrometry (LC/MS) (Synapt G2 HDMS, Waters Corp., Manchester, UK) as previously described (43). For the anti-oxidative and anti-inflammatory functions of HDL, superoxide production and VCAM-1 expression were respectively quantified as previously described (14).

Genotyping of cohorts

The Rotterdam, GiraFH and PAS cohorts were genotyped using taqman probes (ABI) following manufacturer's instructions (ABI).

Statistical Analyses

Regression modeling was used to test for association between lipid measurements and the genetic carrier status versus non-carrier status. Proc Glimmix in SAS software version 9.3 was used with a random effect variable to account for the families from which carriers were ascertained. Adjustment for age and sex was used for test of lipid parameters except for HDL percentiles, which already take into account age and sex. We assumed a dominant model, combining the heterozygous and homozygous carriers of the mutations. Fisher exact tests were

used for proportional comparisons. For meta-analyses, a fixed effects model was used and no adjustments for multiple testing of SNPs within each study were used or for familiarity of samples in those tests. Fisher's Z-score was used to capture each study's effect and to generate a summary correlation statistic. Analyses were conducted using Comprehensive Meta Analysis version 2.2.050.

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Conflict of Interest Disclosures: R.S., I.T., J.M. and M.W. are employees and MRH is on the Board of Directors of Xenon Pharmaceuticals Inc. J.J.K. serves as a consultant to and receives research grants from Xenon Pharmaceuticals Inc. J.C, B.H, L.L, T.R, K.W, L.M and M.vH are employees of Merck Research Laboratories. SA and HC are employees of Vascular Strategies, LLC.

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Table 1: *LIPG* mutations identified, their conservation and predicted functional effect

Mutation	Novel	Vertebrate Conservation	Predicted Effect
Met1-18 T>C	Yes	Conserved to lizard	Protein translation reduced
L130F	Yes	Conserved to stickleback	Probably damaging
G196R	Yes	Conserved to stickleback	Probably damaging
H220Q	Yes	Conserved to stickleback	Probably damaging
E388X	Yes	Conserved to stickleback	Probably damaging
E391K	Yes	Gln tolerated	Possibly damaging
N396S	No	Conserved to stickleback	Possibly damaging
R476W	No	Arg, Lys, Gln tolerated	Possibly damaging

Table 2: Number of *LIPG* mutation carriers by mutation

Mutation	Number of mutation carriers
N396S	81
R476W	27
L130F	12
H220Q	4
G196R	3
E388X	2
E391K	1
Met1-18G>T	1

One individual has a H220Q and a N396S mutation

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Table 3: Plasma lipid levels in *LIPG* mutation carriers

	Carriers	Unrelated Controls	1st degree relative controls	p-value[1] carriers vs unrelated controls	p-value[1] carriers vs 1st degree relatives
N	131	756	123		
% male	71/131 (54.2%)	367/756 (48.5%)	53/123 (43.1%)	0.97	0.09
Age (yrs)	43.8 (19.7)	53.2 (13.5)	39.8 (18.8)	0.011	0.13
HDL-C (mmol/L)	1.80 (0.71)	1.40 (0.60)	1.54 (0.52)	0.00481	0.00003
HDL%ile	72.7 (30.5)	48.8 (34.9)	59.2 (30.6)	0.00936	0.00028
LDL-C (mmol/L)	3.26 (1.09)	3.30 (0.97)	3.10 (1.14)	0.4473	0.83075
TC (mmol/L)	5.60 (1.33)	5.32 (1.16)	5.14 (1.28)	0.06301	0.02057
TG (mmol/L)	1.17 (0.72)	1.36 (0.98)	1.07 (0.67)	0.54617	0.63620
BMI (kg/m^2)	23.8 (3.9)	25.7 (4.0)	23.1 (5.1)	0.32677	0.93010

[1]General linear mixed model with a random variable to account for family membership and adjustment for age and sex when appropriate. Values are: average (standard deviation).

Table 4: HDL-C elevation in partial and complete loss of function *LIPG* mutation carriers

	N	HDL-C	HDL-C increase	% HDL
Unrelated controls	756	54.1(23.2)		
N396S	80	68.1(28.2)	14.0	25.9%
R476W	27	66.9(28.2)	12.8	23.7%
Complete LOF	23	77.3(21.5)	23.2	42.9%
Compound heterozygote	1	108.7	54.6	100.9%
N396S homozygotes	2	94.7(2.7)	40.6	75.0%
p-value (N396S vs controls)		5.50E-07		
p-value (R476W vs controls)		0.005		
p-value (CLOF vs controls)		3.6E-06		
p-value (N396S homozygotes vs controls)		0.3		

HDL-C and HDL-C increase are in mg/dL. Values are average (standard deviation).

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Table 5: *LIPG* mutation carriers with atherosclerosis

	N	*LIPG* carriers with CAD	Controls with CAD	p-value
Family cohort (total)	887	14/131 (10.7%)	111/756 (14.7%)	0.28
Family cohort (PLOF)		12/108 (11.1%)	111/756 (14.7%)	0.38
Family cohort (CLOF)		2/23 (8.7%)	111/756 (14.7%)	0.56
Rotterdam	5814	17/156 (10.9%)	752/5658 (13.3%)	0.37
GiraFH	2068	11/57 (19.3%)	579/2011 (28.8%)	0.12
	N	*LIPG* carriers in CAD cohort	*LIPG* carriers in control cohort	p-value
PAS/Sanquin	665/1078	14/665 (2.10%)	34/1078 (3.15%)	0.24

Controls = Non-*LIPG* mutation carriers. Values are average (standard deviation).

Table 6: Meta analysis of *LIPG* mutation carriers with atherosclerosis

Cohort	Carriers with CAD	Carriers without CAD	Controls with CAD	Controls without CAD	OR	Lower limit	Upper limit	Fisher's Z	p-value
Rotterdam	17	139	752	4875	0.79	0.48	1.32	-0.89	0.37
GiraFH	11	46	579	1432	0.59	0.30	1.15	-1.55	0.12
PAS	14	34	651	1046	0.66	0.35	1.24	-1.29	0.20
Fixed Effect					**0.696**	**0.496**	**0.979**	**-2.083**	**0.037**

Figure Legends:

Figure 1: Functional characterization of LIPG mutations. (A) A schematic of the EL protein with the locations of the mutations indicated. Mutations were found throughout the EL protein. **(B)** Quantification of EL activity in transfected cell culture supernatants using a fluorescent

phospholipid lipase assay (n=3, each n in duplicates). All *LIPG* mutations showed decreased EL activity compared to wt, with mutations in the lipase domain displaying only ~50% inhibition of EL function, whereas all other *LIPG* mutations showed complete loss of EL activity. **(C)** Plasma HDL cholesterol levels in control (WT, n=756), heterozygous and homozygous partial loss of function, heterozygous complete loss of function, and a compound heterozygous individual are graphed against the projected inhibition of EL in the mutation carriers, based on the *in vitro* activity of the mutations. **(D)** The number of individuals with HDL-C<80th percentile was increased in the partial LOF mutation carriers (N396S and R476W) compared with carriers of the complete LOF mutations.

Figure 2: Functionality of HDL from LOF LIPG mutation carriers. (A) ApoB-depleted serum from complete LOF mutation carriers showed a significantly increased macrophage cholesterol efflux acceptor capacity, whereas partial LOF carriers were no different from controls. Wt: n=4; PLOF: n=9; CLOF: n=5. **(B)** NMR analysis showed a significant increase in HDL particle size in complete and not partial LOF *LIPG* mutation carriers. Wt: n=4; PLOF: n=9; CLOF: n=6) **(C)** NMR analysis also showed a significant increase in the concentration of large HDL particles in complete and not partial LOF mutation carriers Wt: n=4; PLOF: n=9; CLOF: n=6) **(D)** By 2-D gel electrophoresis, a significant increase in large α-1 HDL particles was observe in complete LOF mutation carriers. Wt: n=5; PLOF: n=9; CLOF: n=4. **(E)** The concentration of phosphatidylcholine was increased in HDL isolated from *LIPG* mutation carriers, but did not reach significance. Wt: n=4; PLOF: n=8; CLOF: n=6. **(F)** HDL from the complete LOF carriers was significantly enriched in longer chain phospholipids, whereas no significant differences were observed in partial LOF carriers. Wt: n=4; PLOF: n=8; CLOF: n=6.